The non-hallucinogenic serotonin 1B receptor is necessary for the antidepressant-like effects of psilocybin in mice

Sixtine Fleury, Katherine M. Nautiyal

Dartmouth College, Hanover, NH 03755

Email: Katherine.nautiyal@dartmouth,edu

Abstract

Recent studies highlight the promising use of psychedelic therapies for psychiatric disorders, including depression. The persisting clinical effects of psychedelics such as psilocybin are commonly attributed to activation of the serotonin 2A receptor (5-HT2AR) based on its role in the acute hallucinatory effects. However, the active metabolite of psilocybin binds to many serotonin receptor subtypes, including the serotonin 1B receptor (5-HT1BR). Given the known role of 5-HT1BR in mediating depressive phenotypes and promoting neural plasticity, we hypothesized that it mediates the effects of psilocybin on neural activity and behavior. We first examined the acute neural response to psilocybin in mice lacking 5-HT1BR. We found that 5-HT1BR expression influenced brain-wide activity following psilocybin administration, measured by differences in the patterns of the immediate early gene c-Fos, across regions involved in emotional processing and cognitive function, including the amygdala and prefrontal cortex. Functionally, we demonstrated that 5-HT1BR is necessary for the acute and persisting behavioral effects of psilocybin. Although there was no effect of 5-HT1BR expression on the acute head twitch response, mice lacking 5-HT1BRs had attenuated hypolocomotor responses to psilocybin. We also measured the persisting antidepressant-like effects of psilocybin using transgenic and pharmacological 5-HT1BR loss-of-function models and found that 5-HT1B was required for the decreased anhedonia and reduced anxiety-like behavior. Finally, using a network analysis, we identified neural circuits through which 5-H1BR may modulate the response to psilocybin. Overall, our research implicates the 5-HT1BR, a non-hallucinogenic serotonin receptor, as a critical mediator of the behavioral and neural effects of psilocybin in mice.

Significance Statement

Psilocybin, the active ingredient of ‘magic mushrooms’ has emerged as a potential fast-acting and long-lasting antidepressant. As a classic psychedelic, it is unclear what mechanisms of actions underlie its antidepressant effects or if the subjective conscious experience is necessary. While 5-HT2AR-mediated effects are most commonly implicated given its role in the hallucinogenic effect, the polypharmacology of psilocybin is likely critical for its complex behavioral effects. Our studies show the necessity of a non-hallucinatory serotonin receptor, 5-HT1BR, in the antidepressant response to psilocybin, and highlight a potential target for the development of effective non-hallucinogenic pharmacotherapies for the treatment of depressive disorders.

Introduction

In recent years, almost 10% of adults in the US meet the criteria for a mood disorder, and nearly 20% for an anxiety disorder (1). Despite the widespread use of selective serotonin reuptake inhibitors (SSRIs) as antidepressants and anxiolytics, these treatments require long-term administration and fail to provide relief for a significant proportion of patients (2). Developing novel therapeutic targets which include rapid-acting effective therapies is important for the treatment of depressive and anxiety disorders. Psychedelic compounds are emerging as potential therapeutic agents for treating psychiatric disorders. Growing evidence supports their efficacy in treating major depressive disorder (MDD) and other mood and anxiety disorders (3–8). Psilocybin is a serotonergic psychedelic found in *Psilocybe* mushrooms which is gaining traction as a promising therapeutic agent (9). Clinical studies report that one to two doses of psilocybin can produce antidepressant effects in MDD patients that persist over one year (5,7,10–13). However, the hallucinogenic properties of psilocybin limit its use for some patient populations and in everyday clinical practice. Uncovering the neural mechanisms responsible for the therapeutic benefits in preclinical studies is therefore crucial to developing other treatment strategies (14,15).

The persisting behavioral effects have been largely attributed to the activation of the 5-HT2A receptor, based on its well-documented role in mediating the acute hallucinogenic properties of serotonergic psychedelics (16–20). However, there is conflicting evidence emerging from preclinical studies on the necessity of 5-HT2AR in the behavioral effects, especially related to depressive behavior using pharmacological and genetic loss-of-function models (21–26). Some recent studies suggest that these behavioral and neural effects may be independent of 5-HT2AR activation (22,26). Psilocybin's capacity to drive neuroplastic changes has been demonstrated through structural changes in dendritic spines, including increased spine density and size in the prefrontal cortex and synaptic changes in the hippocampus (27) —all alterations which may remain intact with blockade of psilocybin binding to 5-HT2A receptors (22,28). Recent studies also implicate the neurotrophin receptor TrkB and brain-derived neurotrophic factor (BDNF) signaling in mediating psilocybin-induced synaptic plasticity, independent of 5-HT2ARs (29).

Psilocybin's active metabolite, psilocin, binds with high affinity to many serotonin receptor subtypes, not just 5-HT2A, and its polypharmacology is likely important for its clinical efficacy (30). One receptor besides 5-HT2A that binds psilocybin and may be important for the effects of psilocybin on behavior is the serotonin 1B receptor (5-HT1BR). As an inhibitory Gi/o-coupled G protein-coupled receptor (GPCR) that inhibits neurotransmitter release at axon terminals, it has been implicated in a number of plasticity mechanisms including long-term potentiation of cortical inputs to the hippocampus, and long-term depression of corticostriatal synapses (31,32). 5-HT1BRs have also been linked to increased anxiety and depressive behaviors (33–35). Finally, 5-HT1BR has been implicated in mediating the effects of other hallucinogens including MDMA and Ketamine (36–40). These data suggest that 5-HT1B could be a critical mediator of psilocybin's persisting therapeutic effects.

Here, we hypothesize that psilocybin modulates depressive-like behaviors through 5-HT1B receptor activation. Using a genetic and pharmacological loss-of-function models, we sought to investigate the role of 5-HT1B in neural and behavioral effects of psilocybin

in mice. We assessed the role of 5-HT1BR in modulating the acute drug effects of psilocybin on brain-wide neural activity and head twitch and locomotor behavior. We also investigated the impact of 5-HT1BR on the long-term behavioral effects of psilocybin on anxiety and anhedonia phenotypes, key features of depressive disorders. Our findings suggest that the 5-HT1BR influences brain-wide neural changes following psilocybin administration and is necessary for its enduring antidepressant-like effects in mice

Results

Psilocybin-induced changes in neural activity require 5-HT1BR expression

To investigate how psilocybin changes brain-wide neural activity, and whether 5-HT1BR expression influences those changes, we performed a whole-brain c-Fos analysis on male and female mice lacking the 5-HT1BR and their littermate wild-type (WT) controls. Brains were collected 2h following a single injection of psilocybin (5mg/kg) or saline vehicle, and we found that the number of c-Fos+ cells was relatively consistent across animals within brain regions (Fig 1A). Overall, there was a significant effect of psilocybin on neural activity measured by c-Fos staining (main effect of psilocybin: $F(1,23)=5.708$, $p=0.026$). Interestingly, the neural activity was significantly influenced by 5-HT1BR expression (genotype by drug interaction: $F(1, 23)=5.986$, $p=0.023$). Some brain regions showed significant effects of psilocybin regardless of 5-HT1BR expression, such as the somatosensory cortex and the claustrum (CLA) which both express high levels of 5-HT2ARs (Fig 1B, main effect of drug on CLA: $F(1, 22)=21.74$, $p=0.0001$). There were also select brain regions, such as the basolateral amygdala (BLA) which showed differential baseline c-Fos activity based on genotype (main effect of genotype: $F(1, 22) = 4.832$, $p = 0.039$) which potentially drove the differential effect of psilocybin between genotypes (genotype by drug interaction: $F(1, 22) = 11.13$, $p = 0.003$). Most interestingly, quite a few other brain regions showed an effect of 5-HT1BR expression on the c-Fos response to psilocybin including prefrontal cortical areas and the central amygdala (CEA) which showed increased c-Fos in WT, but not 5-HT1BR KO mice (drug by genotype interaction for CEA: $F(1, 23)=8.084$, $p=0.009$; controls: $p=0.0002$, 5-HT1BR KOs: $p=0.669$). The globus pallidus showed a similar pattern with increased c-Fos expression in WT, but not 5-HT1BR KO mice (drug by genotype interaction for GP: $F(1, 22)=4.954$, $p=0.037$; WT: $p=0.003$; 5-HT1BR KO: $p=0.829$). Importantly, these regions express high levels of 5-HT1BR protein (41,42), and may be key sites for psilocybin acting via these receptors to influence neural activity. Overall, psilocybin differentially influenced neural activity throughout the brain leading to both increases and decreases depending on the brain area (main effect of region: $F(28,628)=7.26$, $p<0.0001$), and this pattern of neural activity was differentially influenced by 5-HT1BR expression (Fig 1C; genotype by drug by region interaction: $F(28, 628) = 2.352$, $p=0.0001$). This points to a role for the 5-HT1BR to mediate the behavioral effects of psilocybin.

5-HT1BR is involved in some of the acute behavioral effects of psilocybin

To investigate the functional effect of 5-HT1BR activation on the acute behavioral response to psilocybin, we measured the head twitch and locomotor response to psilocybin in male and female WT and 5-HT1B KO mice. Mice treated with psilocybin demonstrated significantly more head twitch responses than mice treated with saline (Fig 2A; main effect of psilocybin: $F (1, 28) = 312.3$). Additionally, there was no

significant difference in the number of head twitches between genotypes (main effect of genotype: $F(1, 28) = 0.4982$). This indicates that psilocybin robustly induces head twitches in mice independent of 5-HT1BR, which is consistent with previous research implicating 5-HT2AR activation in this acute response to psilocybin. We also analyzed the locomotor response in mice lacking 5-HT1BR expression, and found that unlike the head twitch response, the 5-HT1BR may be involved in the acute hypolocomotor effects of psilocybin (Fig 2B). Specifically, in the first 30min following psilocybin, mice showed a significant decrease in locomotion (main effect of psilocybin: $F(1, 28) = 5.514$, $p=0.026$). This effect seemed attenuated in mice lacking 5-HT1BR, however it wasn't statistically significant (genotype by psilocybin interaction: $F(1, 28) = 1.1321$, $p=0.260$). Within genotype planned comparisons showed significant reductions in locomotor activity in WT ($t(16)=2.666$, $p = 0.0169$), but not in 5-HT1BR KO mice ($t(12) = 0.79$, $p = 0.445$). Overall, these data show that 5-HT1BR mediates some acute behavioral responses to psilocybin, though not the classic head twitch response.

5-HT1BR is required for antidepressant-like effects of psilocybin

Given that there are long lasting behavioral effects of a single administration of psilocybin in humans, we next wanted to examine the role of the 5-HT1BR in the persisting behavioral effects in mice. We administered chronic corticosterone in the drinking water for four weeks to induce a stress-phenotype to measure antidepressant-like effects of psilocybin (Fig 3A). We tested mice lacking 5-HT1BR expression and littermate WT controls in a series of behavioral tests that evaluated anhedonia and anxiety-like behaviors, performed 24-72h after a single i.p. injection of either vehicle or 5mg/kg psilocybin. Interestingly, in male mice, we were unable to find any effect of psilocybin in these tests following chronic corticosterone treatment (see Fig S2) and followed up later with an alternative chronic behavioral despair stressor in males. However, in female mice, psilocybin significantly increased hedonic responding in the gustometer as measured by increased licking for sucrose (Fig 3B; main effect of psilocybin: $F(1,48) = 7.437$, $p = 0.009$). There was also a significant effect of genotype ($F(1, 48) = 12.88$, $p = 0.0008$) which is consistent with the baseline differences between genotypes that we have previously reported (43). Given this, we compared the effect of psilocybin within each genotype. While WT mice showed a significant elevation in sucrose consumption (Fig 3C; sucrose concentration by psilocybin interaction: $F (10, 160) =1.980$, $p=0.039$), there was no effect of psilocybin in female mice lacking 5-HT1BR (Fig 3D; sucrose concentration x psilocybin interaction: $F(5, 115) = 0.445$, $p=0.816$). Overall, these data suggest that psilocybin may influence anhedonia in mice through actions at the 5-HT1BR.

Expression of 5-HT1BR also significantly modulated the anxiolytic response to psilocybin. First, we measured anxiety-like behavior in the conflict-based novelty-suppressed feeding (NSF) assay 48h following psilocybin administration. Interestingly, there was an anxiolytic response to psilocybin that was significantly blunted in the absence of 5-HT1BR expression (genotype by drug interaction: $F(1,49) = 4.072$, $p = 0.049$). Specifically, WT mice injected with psilocybin showed reduced latency to eat compared to saline-treated mice (Fig. 3E, $\chi^2 = 9,615$, $p = 0.002$), while we saw no significant effect in the 5-HT1BR KO mice (Fig. 3F, $\chi^2 = 1.850$, $p = 0.174$). We also tested mice in the elevated plus maze (EPM), as a second measure of anxiety-like behavior, 72h following psilocybin administration. We again found that psilocybin-induced decreases in anxiety behavior were dependent on 5-HT1BR (Fig. 3G; main

effect of drug by genotype ($F(1, 51) = 4.080$, $p = 0.0487$). Psilocybin administration significantly decreased anxiety in female WT mice, as measured by increased entries in open arms ($p=0.01$), but not in mice lacking 5-HT1BR ($p = 0.732$). Overall, convergent evidence from two conflict-based assays shows that psilocybin's persisting anxiolytic response requires 5-HT1BR.

Given that we administered chronic corticosterone to mice to induce a stress phenotype, we assessed if the 5-HT1BR-dependent effects were due to a differential response to corticosterone, rather than a differential response to psilocybin. There were no genotype effects of chronic corticosterone on anxiety behavior in the EPM, with both genotypes showing decreased entries into the open arms (Fig S3A; main effect of genotype: $F(1, 68) = 0.149$, $p = 0.7001$). This suggests that the genotype difference in the psilocybin effect on anxiety-like behavior is not due to a differential response to the stressor. Overall, these behavioral results indicate that in female mice, psilocybin produces an antidepressant-like phenotype in the days following administration which we measured as decreased anhedonia in the gustometer and decreased anxiety-like behaviors in the NSF and EPM. Interestingly, these behavioral effects were attenuated in the absence of 5-HT1BR expression, suggesting that the antidepressant-like effects of psilocybin are dependent on 5-HT1BR following chronic corticosterone administration in female mice.

Additionally, to investigate if the behavioral effects are due to activation of the 5-HT1BR during the acute phase of psilocybin, and to circumvent genotype differences seen in a couple of the behavioral tests, we performed another experiment in which we pharmacologically blocked 5-HT1BR with pre-treatment with a 5-HT1BR antagonist, GR127935, during psilocybin administration in WT mice. Psilocybin again decreased anxiety-like behavior as measured by increased open-arm entries (Fig. 3H; $t(19)=2.34$, $p=0.031$). However, when pre-treated with GR127935, the effect of psilocybin was blocked ($t(16)=0.119$, $p=0.912$). This suggests that blockade of 5-HT1BR during the acute psilocybin response is sufficient to block the post-acute anxiolytic effects of psilocybin seen in the EPM.

Given that chronic corticosterone administration was not an effective model to measure the persisting behavioral effect of psilocybin in male mice, we turned to a chronic behavioral despair paradigm to test the role of 5-HT1BR in the anxiolytic effects of psilocybin in males. In this paradigm, we found that psilocybin increased open-arm entries in the EPM in WT males (Fig. 3I; $t_{38}=2.130$, $p=0.04$), but not in males lacking 5-HT1BR expression ($t_{16}=0.103$, $p=0.919$). This suggests that psilocybin can also induce anxiolytic effects in males, as well as across multiple mouse models for depression.

Neural circuits influenced by psilocybin are influenced by 5-HT1BR expression

To identify the neural circuits through which 5-HT1BR may mediate the effects of psilocybin on behavior, we performed a network analysis on neural activity following psilocybin to probe functional connectivity. Returning to the c-Fos data (shown in Fig 1), we generated pairwise correlation matrices of brain region activity as measured by number of c-Fos+ cells. A difference matrix for each genotype, representing the change in correlated activity following psilocybin administration, illustrates how the functional connectivity throughout the brain is changed by psilocybin and differs based on 5-HT1BR expression (Fig. 4A). Comparing these two correlation matrices reveals statistically distinct patterns between WT and 5-HT1BR KO mice (Fig 4A; rho = -0.01, p

= 0.80), with WT mice showing more desynchrony following psilocybin, which is diminished in mice lacking 5-HT1BR. We then examined which psilocybin-responsive network was most influenced by 5-HT1BR expression and found significant effects of 5-HT1BR on correlated activity between the central amygdala and the anterior cingular cortex (z=2.00, p=0.05). Widening the confidence interval, we found suggestive differences in the correlated activity of the central amygdala with the claustrum, amongst amygdalar regions, and between the globus pallidum and lateral amygdala and anterior cingulate cortex (Fig 4B). Overall, this network analysis shows that 5-HT1BR expression significantly influences neural circuits that are relevant to the effects of psilocybin on cognition and mood. Furthermore, it points to potential neural mechanisms through which 5-HT1BR may influence the persisting antidepressant effects of psilocybin.

Discussion

Our results highlight a critical role for 5-HT1BR in the response to psilocybin in mice. We found that the absence of this non-hallucinogenic receptor impacts the neural response to psilocybin, as well as the acute and persisting behavioral effects. Using c-Fos as a marker of neural activity, we find increased activity in many regions throughout the brain including in sensory and prefrontal cortices, claustrum, amygdala, hypothalamus, and basal ganglia, which is consistent with previous reports (21,44–46). Interestingly, we saw that mice lacking expression of 5-HT1BR have notable differences in the pattern of neural activity following psilocybin administration, including in prefrontal, hippocampal, and amygdalar areas, all brain regions that express high levels of 5-HT1BR. Functionally, a lack of 5-HT1BR had no effect on the head twitch response, though it did significantly attenuate the acute hypolocomotor effects of psilocybin. Importantly, there were also effects of 5-HT1BR on the persisting behavioral effects of psilocybin. Specifically, the amelioration of stress-induced anhedonia and anxiety-like behaviors following psilocybin was absent in mice lacking 5-HT1BR expression. Overall, this shows that 5-HT1BR expression is necessary for the neural and behavioral effects of psilocybin.

Though we find that 5-HT1BR is required for the persisting effects of psilocybin, our data do not address its sufficiency. The profound neural and behavioral effects of psilocybin are likely supported by its polypharmacology at multiple serotonin receptors (30). Although there is some evidence questioning the necessity of the 5-HT2AR in the behavioral effects of psilocybin in mice (22,25), there are mixed findings on this topic with other reports showing that 5-HT2AR expression is necessary for the anti-depressant effects of psilocybin (21). Tangential to this debate, our results show that acute activation of the 5-HT2A receptor by psilocybin is insufficient to rescue depressive-like behaviors in the absence of the 5-HT1BR.

There are many lines of evidence linking the 5-HT1BR to antidepressant function as well as to the behavioral and neural response to hallucinogens (47,48). As an inhibitory $G_{i/o}$-coupled receptor, its activation reduces calcium influx in axon terminals of serotonin (autoreceptors) and non-serotonin (heteroreceptors) neurons decreasing neurotransmitter release (49–51). Past research shows that 5-HT1BR agonists promote antidepressant-like effects and can increase the rewarding effects of cocaine and social interaction (52–55). 5-HT1BR also interacts with p11 to regulate depression states (56,57). Additionally, 5-HT1BR is necessary for the behavioral and hippocampal

plasticity effects of fluoxetine (31). In addition to mediating depressive phenotypes, there is also evidence implicating the 5-HT1BR in antidepressant response to ketamine (37,40), and in other behavioral effects of MDMA (36,38,39).

In keeping with our previous work showing that the 5-HT1BR has a known role in mediating reward and anxiety behavior (34,43,58), we do find baseline differences in some of the behaviors we tested in 5-HT1BR KO mice. This leaves open the limitation of potential behavioral and mechanistic ceiling effects which could have obscured the effects of psilocybin in this strain. This would be possible either through maxing out the range of the behavioral assays and/or through saturation of the 5-HT1BR-dependent mechanism through which psilocybin has its effects. We first addressed these limitations by using at least one behavioral assay with no baseline differences, namely the elevated plus maze. Additionally, we tested mice with adult knockdown of the receptor to eliminate any developmental compensation. Finally, we used a pharmacological blockade of 5-HT1BR by administering an antagonist prior to psilocybin administration. This not only allowed us to circumvent any baseline differences, but also helped identify that 5-HT1BR is involved during the acute drug effect.

Consistent with past research, we find some sex differences in our behavioral effects of psilocybin. Recent work shows that psilocybin differentially influences hypothalamic reactivity to aversive stimuli and reduces ethanol consumption specifically in male, but not female rodents (44,59). On the other hand, females display more head twitch behavior to the psychedelic 1-(2,5-dimethoxy-4-iodophenyl)-2-aminopropane (DOI) and less of an augmentation of pre-pulse inhibition in males, in a strain dependent manner (60,61). The sex differences that we see in our behavioral data may have emerged from a differential response to the stress paradigm rather than a differential response to psilocybin. Past work shows that there are sex-specific effects of chronic corticosterone administration in the drinking water (62,63), and we found that psilocybin ameliorated the effects of corticosterone in females but not males. However, using an alternative forced-swim stressor, we were able to see effects of psilocybin on anxiety-like behavior in males. This suggests that different stress modalities may induce sex-dependent alterations that are amenable to varied pharmacological interventions. Ultimately, there appears to be a substantial impact of sex on the effect of psilocybin on behavior, which are dependent on strain and paradigm, reinforcing the importance of examining sex differences in future studies.

While the 5-HT2AR plays a central role in psilocybin's acute psychedelic effects, its therapeutic potential in the context of stress-induced depression may be limited without the support of other serotonin signaling pathways, including those acting via 5-HT1B. The absence of 5-HT1BR signaling may prevent psilocybin from achieving its full therapeutic potential as the feedback inhibition of serotonin release is disrupted, leading to overactivation of serotonergic circuits, and limiting the ability to modulate neural mechanisms effectively. This could result in a mechanistic ceiling of 5-HT2A, essentially maxing out the 5-HT2A-dependent effects on anhedonia and anxiety-like behaviors. This hypothesis arises in part from studies showing increased 5-HT2AR expression following chronic exposure to stress (64–66). However, neither stress nor the knock-out model seem to have affected acute 5-HT2AR activation in our study, as measured by head twitch responding. Taken together, these results highlight that psilocybin's persisting effects on behavior may depend on an optimal balance of serotonin tone acting across different serotonin receptor subtypes.

Our studies begin to point to the anterior cingulate cortex, the claustrum, and the amygdala as potential targets of psilocybin which are modulated by 5-HT1BR expression. Some preclinical studies have already highlighted the amygdala, especially the basolateral and central nucleus of the amygdala, as important loci for the therapeutic effects of psilocybin (67–70). Given the role of the amygdala in mediating the effects of psilocybin on threat responding (71), it is possible that 5-HT1BR expression on amygdalar terminals underlies our reported behavioral effects. We also found that the 5-HT1BR-effects on functional connectivity seemed to involve the amygdala as a node given its high number of connections with other brain regions that were modulated by 5-HT1BR expression. Our current studies are focusing on region-specific knockdown of 5-HT1BRs in the amygdala to test their functional role in modulating the effects of psilocybin on emotional reactivity. Projection specific studies will also investigate if prefrontal-amygdala networks are involved, and how psilocybin may influence these connections via 5-HT1BR since serotonin is known to gate cortical glutamatergic inputs into the BLA via 5-HT1B receptors (72). Understanding how psilocybin affects interregional connectivity within these networks via its actions on 5-HT1BR activation will help identify the mechanisms through which psilocybin exerts its antidepressant effects.

Overall, our work highlights the complexity of the serotoninergic targets involved in the therapeutic efficacy of psilocybin. Specifically, these studies add to our understanding of the importance of non-5-HT2AR targets by implicating 5-HT1BRs in the antidepressant-like effects of psilocybin in mice. These data contribute to the broader understanding of the polypharmacology of psilocybin and highlight the need for multi-receptor approaches to better understand psilocybin's mechanisms of action. Additionally, our data demonstrate a dissociation of the acute head twitch response from the longer-term effects of psilocybin, suggesting that different mechanisms of actions may underlie the acute psychedelic effect of psilocybin and the persisting behavioral effects. We suggest that psilocybin's effects are not solely driven by 5-HT2AR activation, and that activation of other serotonin receptors are critical for its therapeutic potential. These results also raise the potential for development of effective non-hallucinogenic pharmacotherapies targeting 5-HT1BR for the treatment of depressive disorders.

Materials and Methods

Animals. Mice were bred in the Dartmouth College vivarium and weaned at postnatal day (PN) 21 into cages of 2–4 same-sex littermates. Cohorts of mice lacking 5-HT1BR expression and littermate controls were generated by crossing female homozygous floxed-tetO1B mice to male homozygous floxed-tetO1B mice with the βActin-tTS transgene (tetO1B+/+ females crossed to tetO1B+/+::βActin-tTS + males), as previously reported in (58). N=27 (11 males, 16 females) were used in the c-Fos study. N=32 mice (16 females, 16 males) were used to assess the acute behavioral effects of psilocybin. N=118 (44 males, 74 females) were used to assess persisting behavioral effects of psilocybin following chronic corticosterone administration. N=57 males were used to assess persisting behavioral effects of psilocybin following chronic behavioral despair. N = 39 female mice were used for antagonist pre-treatment. For the adult rescue experiment (N=28), temporal-specific knockdown was achieved by administration of doxycycline to tTS lines, as previously reported in (58). All mice were maintained on a 12:12 light-dark cycle and on *ad libitum* chow and water except as noted below for treatments and behavioral testing. All procedures were approved by the Dartmouth College Institutional Animal Care and Use Committee.

Drugs and treatments. Psilocybin (Cat # 14041, Cayman Chemical), or saline vehicle, was injected intraperitoneally (i.p.) at the dose of 5mg/kg diluted in saline solution (0.9% NaCl) at a volume of 10ml/kg. For antagonist experiments, 0.9% saline vehicle, or the serotonin 5-HT1B/1D receptor antagonist GR127935 (CAT#508014; Sigma-Aldrich) was injected i.p. at 10mg/kg (in 0.9% saline at10mg/kg) 30 minutes prior to vehicle or psilocybin administration.

Chronic corticosterone administration. Corticosterone (35 µg/mL; Cat # 2505, Sigma-Aldrich) was dissolved in 0.45% beta-cyclodextrin (Cat # 4767, Sigma-Aldrich) in drinking water, and delivered *ad libitum* in opaque bottles for 4 weeks. This paradigm produces a corticosterone dosing of approximately 9.5 mg/kg/day (73).

Chronic behavioral despair model. As an alternative to chronic corticosterone administration, a group of male mice were subjected to repeated stress exposures via five forced swim sessions. Each mouse was placed in a 2-liter beaker containing water (22-25ºC) for 5 minutes daily for five consecutive days. Mice remained in their home cages between sessions. Mice were injected with psilocybin (1mg/kg, i.p) or saline vehicle (10 ml/kg) 24 hours after the last swim session.

Behavioral tests.

Head Twitch Response and Locomotion. Head twitches and locomotion were analyzed in a subset of mice after the administration of either vehicle or psilocybin (5mg/kg, i.p) in both control and mice lacking 5-HT1BR. Immediately following injections, mice were placed in an open field, and video recording was initiated with a camera positioned directly above the arena for 1 hour. Three experimenters scored the first 15 minutes of activity for head twitches while blinded to treatment, noting the total number of and time stamp of each head twitch to compare across experimenters. Locomotion was scored with EthoVision software.

Gustometer. A Davis Rig 16-bottle Gustometer (Med Associates MED-DAV-160 M) was used to test the effects of psilocybin and 5-HT1BR expression on hedonic responding. Mice were water-restricted for 5 days of initial training before being put on corticosterone. On day 1 of training, mice were placed in the gustometer as a cage and with free access to 5% sucrose for 15 minutes. On day 2, mice were placed individually with free access to 5% sucrose for 15 minutes. On day 3, mice were placed individually with access to 5% and 10% sucrose with tubes alternating for 30 minutes. Mice were then food restricted for 2 days before being individually returned to the gustometer for 30 minutes with access to water, 2% sucrose, 4% sucrose, 6% sucrose, 8% sucrose, and 10% sucrose for a baseline test of anhedonia using 6 bottle paradigm. Each concentration was randomly presented for a maximum of 120 seconds at a time before switching to another tube. Total licks at each tube were recorded using a capacitance-based system. Mice that had less than 300 licks during the final training session were excluded from the analysis. After the baseline test, mice were put on corticosterone. The 6 bottle paradigm was then repeated 24 hours after drug administration for the experimental gustometer test. Each mouse was weighed before the 6 bottle paradigm and data were normalized by weight. After the experimental test, mice were allowed food that day until 24 hours before the time of the novelty-suppressed feeding test (about ~1h30).

Novelty Suppressed Feeding (NSF). Twenty-four hours following testing in the gustometer, the NSF assay was conducted in a rodent transport container (20' x 16') with the floor covered with approximately 2-4cm of corn bedding to serve as an arena. Thirty minutes before testing, mice were placed individually into holding cages. At the time of testing, a single pellet of food was placed on a white platform (~4' diameter) in the center of the arena. The anxiogenic environment was produced by placing a lamp with high luminosity (~120 lux) above the center of the arena. Each mouse was placed in the same corner of the arena, facing the wall, and a stopwatch was immediately started. The latency to eat, defined as the mouse biting the pellet, was recorded over a session of 5 minutes. Immediately after a bite, the pellet was removed from the arena. To control for hunger, mice were immediately placed in their homecage after the testing session with a new food pellet, and the stopwatch was started again to measure latency to eat within their homecage. Animals that did not bite during the testing session were attributed the maximum value (300 seconds). Animals were placed back in their homecage with food and water ad libitum once all cage mates were tested.

Elevated Plus Maze (EPM). The test was conducted 24 hours after the NSF. Mice were placed in the center facing a closed arm and videotaped while allowed to explore the maze undisturbed for 6 minutes. The maze has four arms (35cm long x 5cm wide) that were 60cm above the ground. The two opposite closed arms had 20cm high walls, while the two opposing open arms had a 1cm lip. Behavior was scored using EthoVision software for the number of entries and time spent in each arm.

c-Fos Whole Brain Analysis

Brain samples. A set of male and female tTS- (N=14) and tTS+ (N=14) mice was used to perform a whole brain c-Fos analysis following vehicle or psilocybin (5mg/kg, i.p) administration. Brain-wide c-Fos expression was induced during the acute phase of psilocybin administration. Mice were injected with either vehicle or psilocybin (5mg/kg), and then placed back in their home cage for 2h, before perfused with 0.1 M PBS followed by 4% formaldehyde. The brain was extracted and postfixed for 24 hours in 4% formaldehyde before being cryoprotected in 30% sucrose. The brain was sectioned in a coronal plane with a thickness of 40um on a cryostat (Leica Biosystems) and stored in 0.1 M PBS at 4ºC in 24 well plates. About 20 to 30 sections per brain were selected for staining.

Tissue processing. Sections were first washed three times in PBS-T (0.1%) for 10 minutes. Sections were then blocked for 1h in 2% Normal Donkey Serum (#NC9624464, Fisher) in PBS-T (0.1%). Sections were then incubated in primary antibody (rb anti-cfos Rabbit Recombinant Monoclonal c-Fos antibody, ab214672) at 1:1000 in blocking solution overnight at 4ºC. The following day, sections were washed 3 times again in PBS-T (0.1%). Sections were then incubated in secondary antibody Alexa Fluor 488 anti-rabbit (ab150073, Abcam) at 1:250 and DAPI at dilution 1:10,000 in PBT (0.15) for 2 hours away from light. Sections were then washed again three times and mounted. Once dry, the slides were cover slipped with ProLong Gold. Whole sections were then imaged on Keyence BZ-X800 microscope with a 10x objective. Fluorophores were imaged with DAPI and GFP filter cubes.

Segmentation of fluorescent labels and quantification. Cells expressing a c-Fos label were segmented using the machine learning-based pixel and object classification program, *Ilastik*, as previously described in Berg et al., 2019. *Ilastik* was also used to generate binary images of the segmented c-Fos labels. Registration and quantification of histological images on a brain-wide scale were performed using the open-source atlas registration tool FASTMAP, as previously described in (74).

Statistical Analyses

All behavioral analyses were performed using GraphPad Prism (v.10.2.2) or RStudio (v.2023.06.1+524). The threshold of statistical significance was set at 0.05. Data were analyzed using one-, two-, three-, and four-way ANOVAs and t-tests using GraphPad Prism, with Tukey's correction for multiple comparisons when appropriate. Functional connectivity analyses were performed on a collection of 29 regions based on our interest and ability to reliably and consistently delineate these regions using a DAPI stained reference image. A psilocybin index was generated by subtracting average total c-FOS counts. The density of regional c-Fos expression was cross-correlated within each group to generate pairwise correlation matrices, and difference matrices were obtained by subtracting coefficients within each element. The similarity and strength of matrices were assessed using nonparametric permutation testing Monte Carlo and Spearman correlation to generate rho and p values. For network analysis, we performed a fisher r-to-z transformation on correlation and selected significant edges at 95%, 90%, 85%, and 80% confidence intervals.

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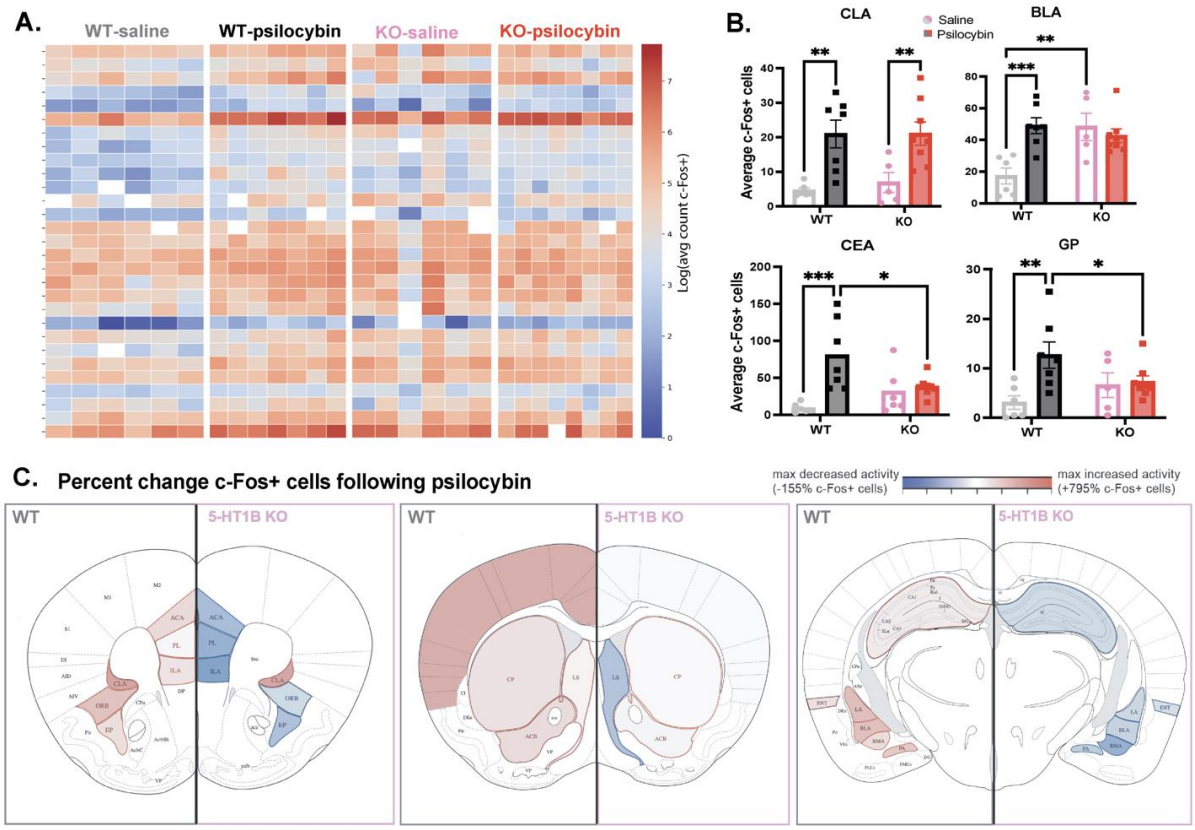

Figure 1: Psilocybin-induced changes in neural activity are dependent on 5-HT1BR
(A) Heatmap shows log-transformed counts of c-Fos+ cells for individual mice (columns) across various brain regions (rows) for WT and 5-HT1BR knockout (KO) mice treated with saline or psilocybin. **(B)** Group means of c-Fos+ cells in the claustrum (CLA), basolateral amygdala (BLA), central amygdala (CEA), and globus pallidus (GP) following psilocybin or saline treatment. **(C)** Percent change in c-Fos+ cells following psilocybin administration is shown in three diagrams of coronal sections of the brain (at bregma +1.94mm, +0.98mm, and -2.18mm) in control (left hemisphere of each diagram) and 5-HT1BR knockout (right hemisphere of each diagram) mice. Psilocybin leads to both increases (shown in red) and decreases (blue) in c-Fos+ cell counts, with notable differences between genotypes, calculated as relative changes in c-Fos expression compared to saline-injected controls. Data are presented as mean ± SEM. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$. ILA: infralimbic cortex, PL: prelimbic cortex; ORB: orbitofrontal cortex, ACA: anterior cingulate cortex, CLA: claustrum, EP: endopiriform nucleus, CP: caudoputamen, ACB: nucleus accumbens, LS: lateral septal area, ENT: entorhinal cortex, LA: lateral amygdala, BLA: basolateral amygdala, BMA: basomedial amygdala, PA: posterior amygdala nucleus

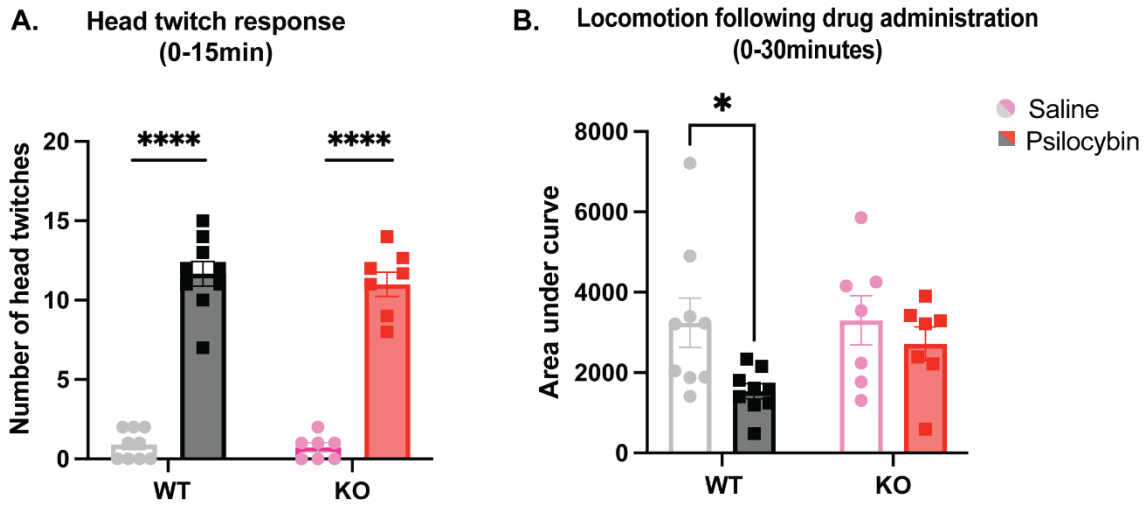

Figure 2: 5-HT1BR is involved in some of the acute effects of psilocybin

(A) Head twitch response is shown for 5-HT1BR KO mice and littermate WT controls over the 15-minute period following administration of saline or psilocybin. Psilocybin treatment significantly increased the number of head twitches in both WT and KO mice compared to saline-treated controls (****$p < 0.0001$), with no significant difference between genotypes. (B) Locomotor activity is shown for 5-HT1BR KO mice and littermate WT controls mice over the 30 minutes following administration of saline or psilocybin. Psilocybin reduced locomotion in WT mice compared to saline-treated mice (*, $p < 0.05$). However, this reduction was not observed in KO mice, indicating an attenuated locomotor response to psilocybin in the absence of 5-HT1BR. Data are represented as mean ± SEM.

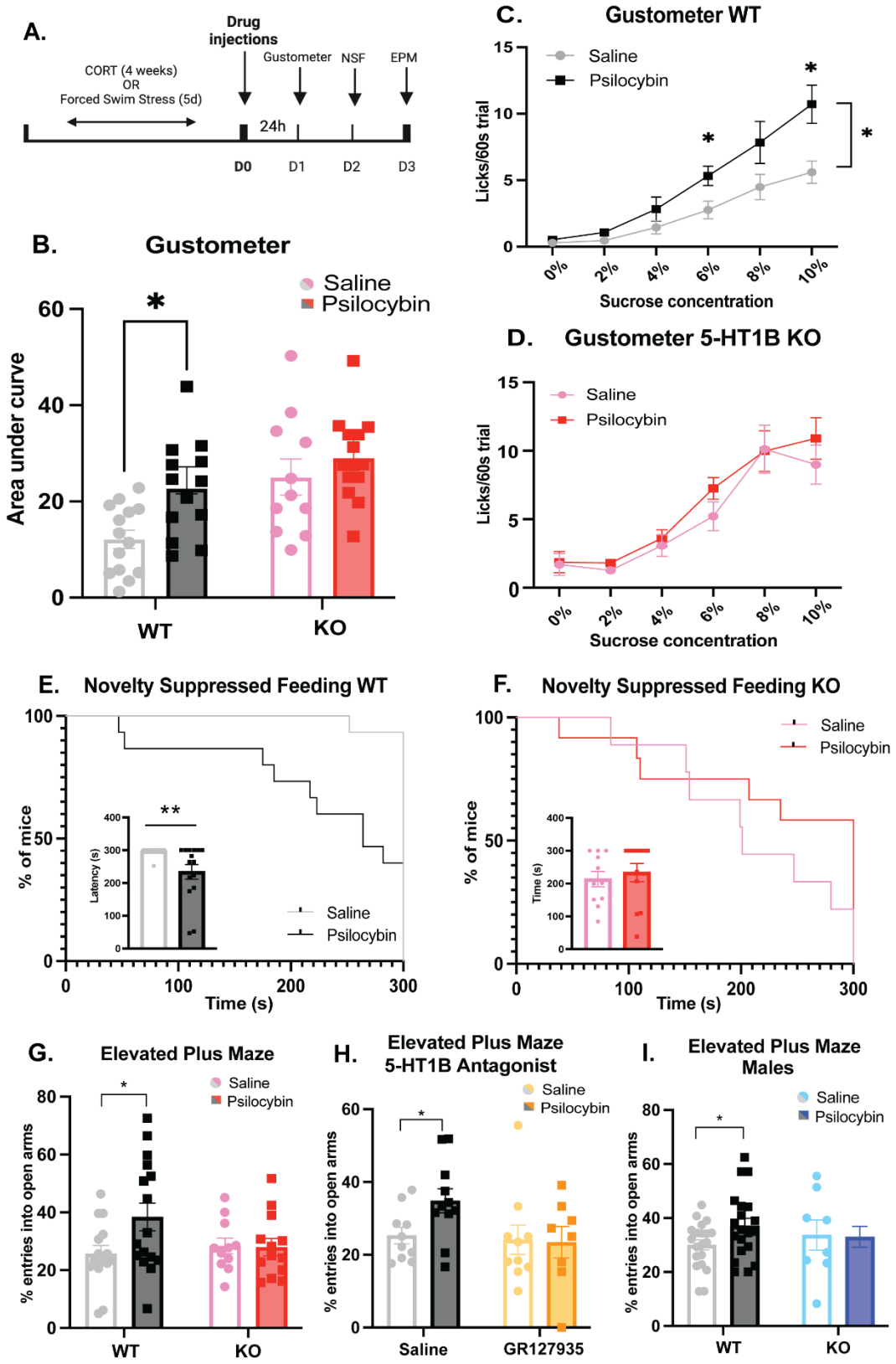
A.
Drug injections
CORT (4 weeks)
OR
Forced Swim Stress (5d)
Gustometer
NSF
EPM
24h
D0
D1
D2
D3
B. Gustometer
Saline
Psilocybin
Area under curve
WT
KO
C. Gustometer WT
Licks/60s trial
Sucrose concentration
D. Gustometer 5-HT1B KO
E. Novelty Suppressed Feeding WT
% of mice
Latency (s)
Time (s)
F. Novelty Suppressed Feeding KO
G. Elevated Plus Maze
% entries into open arms
H. Elevated Plus Maze 5-HT1B Antagonist
Saline
GR127935
I. Elevated Plus Maze Males

Figure 3: 5-HT1BR is required for antidepressant-like effects of psilocybin

(A) Experimental time line is shown for studies testing the post-acute behavioral effects of psilocybin. **(B)** Area under the curve (AUC) analysis of gustometer data showed a significant effect of psilocybin in WT mice (*$p < 0.05$), but not in KO mice. **(C)** Psilocybin increased hedonic responding, as measured by the number of licks in the gustometer in WT mice across increasing sucrose concentrations compared to saline-treated controls (*$p < 0.05$). **(D)** 5-HT1BR KO mice did not show a significant change in hedonic responding following psilocybin treatment compared to saline, indicating a diminished psilocybin effect in the absence of 5-HT1BR. **(E,F)**The Kaplan-Meier survival curves represent the time of first bite (seconds) measured in the Novelty-Suppressed Feeding (NSF) test for WT (E) and 5-HT1BR KO mice (F). Psilocybin significantly reduced latency to feed in WT mice (**$p < 0.01$), indicating decreased anxiety. No significant effect of psilocybin was observed in KO mice. **(G)** In the Elevated Plus Maze (EPM), psilocybin increased entries into open arms in WT mice (*$p < 0.05$), suggesting reduced anxiety, whereas no effect was observed in KO mice. **(H)** Psilocybin significantly increased entries into the open arms of the EPM in saline-treated WT mice (*$p < 0.05$), suggesting reduced anxiety. However, pre-treatment with the 5-HT1BR antagonist GR127935 blocked this effect, indicating the importance of 5-HT1BR activation during the acute phase of psilocybin for its anxiolytic effects. **(I)** In male mice, psilocybin increased open-arm entries in the EPM in WT mice (*$p < 0.05$), but no significant effect was observed in 5-HT1B knockout (KO) males following a forced swim stressor.

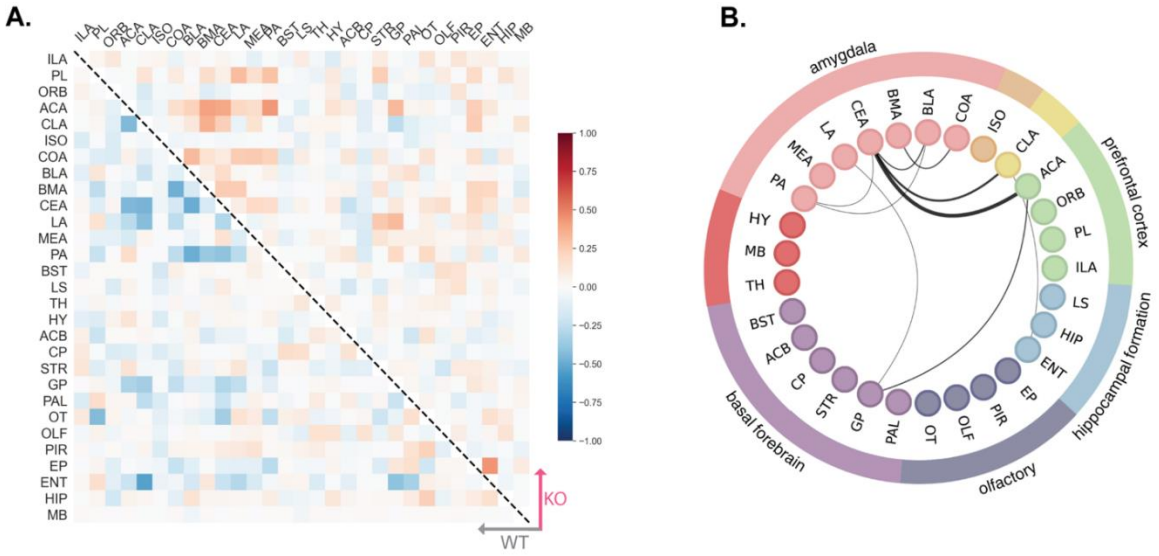

Figure 4: 5-HT1BR expression regulates select neural circuits influenced by psilocybin

(A) The difference correlation matrix shows psilocybin-induced alterations in functional connectivity between WT (lower triangle) and 5-HT1BR KO mice (upper triangle). The matrix represents the pairwise correlation differences across 29 regions, illustrating desynchrony patterns between the two genotypes. WT mice exhibit more desynchrony following psilocybin than 5-HT1BR KO mice do. (B) Circular network plot highlighting psilocybin-responsive connections most influenced by 5-HT1BR expression. Significant changes in correlated activity were observed between the central amygdala (CEA) and the anterior cingulate cortex (ACA) ($p = 0.05$). Expanding the confidence interval to 90%, 85%, and 80% revealed additional differences in connectivity in CEA-claustrum (CLA), ACA-globus pallidus (GP), CEA-cortical amygdala (COA), CEA-basolateral amygdala (BLA), CLA-entorhinal cortex (ENT), GP-lateral amygdala (LA), Posterior amygdala nucleus (PA)-(CEA), (PA)-(BLA). Line width corresponds to the confidence interval, with the thickest edge representing 95% CI.